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PRINCIPAL INVESTIGATOR: Serdar Bulun, M.D.

CONTRACTING ORGANIZATION: The University of Texas Southwestern
Medical Center at Dallas
Dallas, Texas 75235-9106

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Bulun, Serdar, M.D.

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Desmoplastic reaction, *i.e.*, intra- and peritumoral accumulation of fibroblasts provides structural and molecular support for breast tumors. This process of accumulation of fibroblasts around malignant epithelial cells in breast tumors is an active event mediated by malignant cells via endocrine manipulation of the surrounding stromal cells. In this instance, the destiny of the yet undifferentiated adipose fibroblast is the target cell. The secretory products of malignant cells, which have been identified as certain cytokines prevent the differentiation of adipose fibroblasts into mature adipocytes. This is the major mechanism for desmoplastic reaction, since malignant epithelial cells do not induce proliferation of adipose fibroblasts. We also identified the key adipogenic transcription factors that were inhibited by malignant epithelial cells. We are currently evaluating the effects of these factors on the promoter activity of molecules that are important markers for the undifferentiated state of adipose fibroblasts. We are also determining the distribution of expression of adipogenic transcription factors and anti-adipogenic cytokines in breast tumors and surrounding tissues.

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Serdar E. Bulay 10/18/98

PI - Signature Date

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5. INTRODUCTION

This Annual Report was prepared as a requirement of the Idea Award entitled "Adipocyte Differentiation: Relationship to Breast Cancer" funded by the US Army Medical Research and Materiel Command Breast Cancer Research Program (DAMD17-97-1-7025). This report covers research for the period 10/1/97-9/30/98. We will initially provide an overview of this research work. Then, details will be provided in **6. BODY**. The long term objective of this project is to characterize the cellular and molecular mechanisms responsible for intra- and peritumoral accumulation of stromal fibroblasts. To achieve this objective, the following specific aims were proposed. The first **specific aim 1 is** ***to determine whether secretory products of breast cancer cells prevent differentiation of adipose fibroblasts into mature adipocytes.*** Major goals in this aim have been accomplished. First, we established culture systems that permit differentiation of both murine 3T3L1 fibroblasts and human adipose fibroblasts into mature adipocytes. We then demonstrated that coculturing these fibroblasts with breast cancer cell lines prevented the differentiation process. Likewise, culture medium conditioned with breast cancer cells inhibited differentiation of fibroblasts into mature adipocytes. These observations were confirmed using appropriate differentiation markers. **Specific aim 2 is** ***to characterize the secretory products of malignant epithelial cells, which downregulate adipogenic transcription factors.*** This aim has also been accomplished almost completely. First, we determined specific adipogenic transcription factors that are responsible for this tumor effect described above. Then, we demonstrated that TNFα and IL-11 are essential secretory factors of malignant epithelial cells that mediate these effects. **Specific Aim 3 is** ***to determine whether adipogenic transcription factors regulate aromatase P450 expression in human adipose fibroblasts.*** Currently, we are overexpressing C/EBPα, C/EBPβ, C/EBPδ and PPARγ in adipose fibroblasts and determine their effects on P450arom promoter II, which is the preferentially used alternative promoter in breast cancer tissues. These studies have not been completed yet. Finally **specific aim 4 is** ***to determine the regional distribution of C/EBPβ, PPARγ, C/EBPα and P450arom expression in the breast in relation to tumor location.*** We are employing immunohistochemistry to determine the distribution of expression of these adipogenic factors in malignant epithelial cells and in surrounding tissues. We also added the anti-adipogenic IL-11 and TNFα to this repertoire of markers in view of our recent findings. These studies are presently under way.

6. BODY

Malignant breast epithelial cells induce a reaction in the adjacent adipose stroma characterized by accumulation of large numbers of fibroblasts, *i.e.*, desmoplastic reaction. This dense layer of peritumoral fibroblasts arises from the breast adipose tissue and provides structural and biochemical support for breast cancer. Here we seek to determine the epithelial-stromal interactions responsible for desmoplastic reaction using 3T3-L1 murine fibroblasts and human adipose fibroblasts, which differentiate to mature adipocytes as model systems. After exposure to an activating cocktail of hormones for two days, control 3T3-L1 cells differentiated fully to mature adipocytes by days 6-8. Coculturing 3T3-L1 cells with T47D or MCF7 breast cancer cell lines inhibited this differentiation almost completely. Likewise, T47D-cell-conditioned medium gave rise to inhibition of the differentiation of 3T3-L1 cells. T47D-cell-conditioned medium also inhibited the differentiation of human breast adipose fibroblasts in primary culture, whereas control cells differentiated to mature adipocytes. This tumor effect was eliminated using neutralizing antibodies against TNFα or IL-11. TNFα mRNA was demonstrated by northern analysis in T47D cells treated with lipopolysaccharide or TNFα itself but not in 3T3-L1 cells indicating that the malignant epithelial cell is a site of TNFα production. Both immunoreactive TNFα and IL-11 were demonstrated primarily in malignant epithelial cells but not in the surrounding fibroblasts in breast tumors.

Adipocyte differentiation is mediated by the expression of a cascade of adipogenic transcription factors including C/EBPδ or C/EBPβ, PPARγ and C/EBPα. We demonstrated by northern analysis that exposure of 3T3-L1 cells to T47D-cell-conditioned medium did not change C/EBPδ or CEBPβ expression but suppressed or inhibited the expression of PPARγ and C/EPBα in 3T3-L1 cells treated with the cocktail. In these 3T3-L1 cells, inhibition of differentiation was also confirmed by markedly suppressed levels of aP2 mRNA, which is an adipocyte-specific gene. Treatment of 3T3-L1 cells with T47D cell-conditioned medium or TNFα changed neither the numbers of cells in G_O/G_1 or S phases nor the rate of [^{3}H]thymidine incorporation into these cells, thus, ruling out a proliferative effect of malignant cells on the surrounding fibroblasts. In summary desmoplastic reaction primarily occurs via the action of cytokines (TNFα and IL-11) secreted by the malignant epithelial cells to inhibit differentiation of adipose fibroblasts to mature adipocytes. This tumor-induced block in adipocyte differentiation is mediated by the inhibition of PPARγ and C/EBPα expression.

7. CONCLUSIONS

The results summarized above supported the central hypothesis in this grant application. Desmoplastic reaction, *i.e.*, intra- and peritumoral accumulation of fibroblasts provide structural and molecular support for breast tumors. This process of accumulation of fibroblasts around malignant epithelial cells in breast tumors is an active event mediated by malignant cells via endocrine manipulation of the surrounding stromal cells. In this instance, the destiny of the yet undifferentiated adipose fibroblast is the target cell. The secretory products of malignant cells, which have been identified as certain cytokines such as IL-11 and TNFα prevent the differentiation of adipose fibroblasts into mature adipocytes. This is the major mechanism for desmoplastic reaction, since malignant epithelial cells do not induce proliferation of adipose fibroblasts. We also identified the key adipogenic transcription factors that were inhibited by malignant epithelial cells. We are currently evaluating the effects of these factors on aromatase P450 (P450arom) promoter activity in these cells, since P450arom is a marker for the undifferentiated state of adipose fibroblasts and also is responsible for local estrogen biosynthesis in breast tumors. We are also determining the distribution of expression of adipogenic transcription factors and anti-adipogenic cytokines in breast tumors and surrounding tissues.

8. REFERENCES

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IL-11 and TNFα secreted by malignant breast epithelial cells inhibit adipocyte differentiation by downregulating C/EBPα and PPARγ: mechanism of desmoplastic reaction

(*Running Title*: Breast cancer and adipocyte differentiation)

[1]Li Meng, M.D., [2]Jianfeng Zhou, M.D., [3]Hironobu Sasano, [3]Takashi Suzuki, [1]Khaled M. Zeitoun, M.D., and [2]Serdar E. Bulun, M.D.

[1]Department of Obstetrics and Gynecology, University of Texas Southwestern Medical Center at Dallas
[2]Department of Obstetrics and Gynecology, University of Illinois at Chicago
[3]Department of Pathology, Tahoku University School of Medicine, Sendai, Japan

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Address correspondence to:
Serdar E. Bulun, M.D.
Departments of Obstetrics and Gynecology and Molecular Genetics
University of Illinois at Chicago
820 S. Wood St. M/C 808
Chicago, IL 60612

Tel: 312-996-8197
Fax: 312-996-4238
Email: sbulun@uic.edu

ABSTRACT

The dense layer of fibroblasts that accumulate around malignant breast epithelial cells (*i.e.,* desmoplastic reaction) arises from the breast adipose tissue and provides structural and biochemical support for breast cancer. We herein seek to determine the epithelial-stromal interactions responsible for desmoplastic reaction using cultured 3T3-L1 murine fibroblasts and human adipose fibroblasts, which can be activated to differentiate to mature adipocytes, as model systems. Coculturing fibroblasts with various breast cancer cell lines (T47D, MCF7, SSC202, SSC78 and SSC30) or breast cancer cell-conditioned media inhibited this differentiation almost completely, whereas control cells differentiated to mature adipocytes. This tumor effect was eliminated using neutralizing antibodies against TNFα or IL-11. Both immunoreactive TNFα and IL-11 were readily detectable in malignant epithelial cells but not in the majority of the surrounding fibroblasts in breast tumors indicating the malignant epithelial cell is the major site of cytokine production.

Adipocyte differentiation is mediated by the expression of a cascade of adipogenic transcription factors including C/EBPδ, C/EBPβ, PPARγ and C/EBPα. We demonstrated by northern analysis that exposure of 3T3-L1 cells to T47D-cell-conditioned medium did not change C/EBPδ or C/EBPβ expression but suppressed or inhibited the expression of PPARγ and C/EBPα in 3T3-L1 cells treated with the cocktail. In these 3T3-L1 cells, inhibition of differentiation was also confirmed by markedly suppressed levels of aP2 mRNA, which is an adipocyte-specific gene. Treatment of 3T3-L1 cells with T47D cell-conditioned medium or TNFα changed neither the numbers of cells in G_1,G_2 or S phases nor the rate of [^{3}H]thymidine incorporation into these cells, thus, ruling out a proliferative effect of malignant cells on the surrounding fibroblasts. In summary desmoplastic reaction primarily occurs via the action of cytokines (TNFα and IL-11) secreted by the malignant epithelial cells to inhibit differentiation of adipose fibroblasts to mature

adipocytes. This tumor-induced block in adipocyte differentiation is specifically mediated by the inhibition of PPARγ and C/EBPα expression.

INTRODUCTION

The group of infiltrating duct carcinomas constitutes 70 percent of all malignant mammary tumors (1). The neoplastic glandular formations (epithelial cells) are disseminated in a stroma that is comprised of mature adipocytes and fibroblasts. This tumor type, referred to commonly as "scirrhous" because of its extremely hard consistency, contains large numbers of fibroblasts dispersed between malignant epithelial cells, as well as within the immediate periphery of the tumor (desmoplastic reaction). The relationship between adipose stroma and breast cancer is unique in the sense that stromal fibroblasts seem to provide the structural support for cancer growth, whereas malignant cells seem to greatly influence the composition of the adjacent tissue. Evidence from several laboratories indicates that this epithelial-stromal interaction also involves paracrine mechanisms that promote the development and growth of breast carcinomas (2-4). These morphologically identified intra-and peritumoral fibroblasts originate from adipose tissue and most likely represent potential pre-adipocytes, since the fibroblasts isolated from adipose tissue are capable of differentiating to mature adipocytes under defined culture conditions (5,6). Before the current study was initiated, we hypothesized that malignant epithelial cells of breast tumors secrete growth factors and cytokines to prevent the differentiation of fibroblasts to mature adipocytes in the adjacent adipose tissue. The following body of evidence supported this hypothesis. Firstly, tumors were found in breast quadrants with the highest aromatase P450 transcript levels and the highest fibroblast-to-adipocyte ratios (7). This parallelism between the distribution of fibroblasts and aromatase expression is not surprising, since aromatase is a marker for undifferentiated adipose fibroblastic cells (8,9). Secondly, in the cancer-free human breast, the highest fibroblast-to-adipocyte ratios and aromatase P450 transcript levels were found in the lateral and upper regions (10). This distribution roughly correlates with the most common sites where infiltrating duct carcinomas develop. Thirdly, breast tumor-conditioned media was found to induce aromatase expression in adipose fibroblasts (11). This tumor-induced effect can be inhibited and titrated by addition of an anti-IL-11 antibody (our unpublished observations). When aromatase expression is viewed as a fibroblast marker, these results suggested that malignant epithelial cells secrete factors such as IL-11 to prevent differentiation of fibroblasts.

Additionally, estradiol pretreatment of cancer cells potentiated aromatase induction in a dose-dependent fashion, which is suggestive of a paracrine loop (11). Moreover, we demonstrated that certain adipogenesis inhibitors such as IL-11 are secreted by the T47D breast cancer cell line (12). Again, estradiol stimulated IL-11 expression in T47D cells in a dose-dependent fashion (12). Finally, IL-11 (13) and TNFα (14) stimulation of the fibroblast marker aromatase in adipose fibroblasts can be interpreted as inhibition of adipocyte differentiation, since aromatase expression in adipose tissue primarily resides in fibroblasts but not in mature adipocytes (8,9).

There may be multiple potential mechanisms responsible for accumulation of adipose fibroblasts within the tumor and in adjacent stroma. It is possible that fibroblasts proliferate in response to tumor-derived growth factors. Although treatment of murine 3T3-L1 fibroblasts in culture with serum, insulin, IGF-1 and EGF initially causes proliferation; cell replication rapidly stops under these conditions, and fibroblasts eventually differentiate into mature adipocytes (15-18). Thus, it follows that there has to be other effective mechanisms for peritumoral fibroblast accumulation. Inhibition of differentiation or dedifferentiation of preexisting adipocytes may provide these critical mechanisms responsible for extremely high fibroblast-to-adipocyte ratios in the stroma surrounding cancer cells. This process may be under the control of cytokines secreted by the malignant epithelial cells.

The cellular and molecular mechanisms responsible for differentiation of stromal fibroblasts into mature adipocytes were studied intensively (19-24). During mammalian development, embryonic mesoderm gives rise to several highly specialized cell types, including adipocytes. Differentiation of adipocytes from multi-potential fibroblastic precursors appears to be controlled at the transcriptional level by two major groups of tissue-specific transcription factors: the C/EBPα and PPARγ. It has been suggested that C/EBPβ expression initially converts multipotential mesenchymal precursor cells into pre-adipocytes (19). The determined pre-adipocytes were able to respond subsequently to potent adipogenic inducers such as PPARγ. The PPAR isoform, PPARγ (21) is a member of the ligand-activated transcription factor family that heterodimerizes with RXRα and binds to the promoters of adipocyte-specific genes. A third adipocyte-enriched transcription factor, C/EBPα, has been shown to promote the terminal adipocyte

differentiation (20). When expressed together, PPARγ and C/EBPα act synergistically to powerfully promote adipocyte differentiation in fibroblastic cells regardless of tissue of origin (22,24). For practical purposes, we will refer to undifferentiated mesenchymal precursors and determined pre-adipocytes in human adipose tissue as adipose fibroblasts, since both cell types appear as fibroblasts morphologically. Fibroblasts isolated from adipose tissue differentiate into adipocytes when cultured in a defined medium (5,6). On he other hand, certain substances such as TNFα are not only capable of inhibiting adipocyte differentiation, but of reversing it by suppressing the expression of PPARγ (25). Most of the work in this field has been performed using rodent fibroblasts and has been related to obesity and diabetes. The possible roles of malignant epithelial cells in local regulation of these transcription factors and on adipocyte differentiation have not been studied to date. We herein report a series of epithelial-stromal interactions in the breast cancer, which represent the cellular and molecular mechanisms responsible for the development and maintenance of desmoplastic reaction.

MATERIALS AND METHODS

Tissue acquisition.

Breast adipose tissue was obtained from 5 patients undergoing reduction mammoplasty. These tissues were immediately processed for primary cultures of adipose fibroblasts. Breast cancer and surrounding adipose tissue samples were obtained from 25 mastectomy specimens for immunohistological detection of C/EBPs (α, β, δ), IL-1L and TNFα. These studies were conducted following protocols approved by the Institutional Review Boards of the UT Southwestern Medical Center at Dallas and Tohoku University School of Medicine in Sendai, Japan

Detection of transcripts of adipocyte-specific genes and cytokines by RNA blot-analysis.

Total RNA was isolated from fibroblasts/adipocytes in culture, electrophoretically fractionated (10μg) and transferred to a charged membrane. Duplicate measurements of optical density (OD at 260 μm) were performed to equalize loading, which was confirmed by visual inspection of 18S and 28S RNA stained with ethidium bromide. Northern blots were hybridized with cDNA probes labeled by random priming using [^{32}P]dCTP. Complementary DNA templates for adipocyte P2 (aP2), C/EBPδ, C/EBPβ, PPARγ, C/EBPα and TNFα were kindly provided by Drs Steve McKnight, Gokhan Hotamisligil, Carol Mendelson and Bruce Beutler.

Cell cultures and differentiation of fibroblasts to adipocytes.

We routinely perform primary cultures of human adipose fibroblasts as previously described (8). Differentiation of human adipose fibroblasts to mature adipocytes was performed following a modified protocol originally outlined by Hauner and coworkers (5). Breast adipose tissue obtained from women at the time of reduction mammoplasty was processed through the mincing, washing, digestion (with collagenase and centrifugation steps. The floating mature adipocytes were aspirated and the sedimented fibroblast fraction were resuspended in DMEM with 10% fetal calf serum (FCS) as previously described (8). Nucleus-containing cells were inoculated at a density of 50,000/cm^2 into 6-well plates. Cultures were grown for a 24 hour period in DMEM with

10% FCS. Cells were then placed in a chemically defined phenol red-free and serum-free medium consisting of DMEM/Ham's F-12 medium (1:1, v/v), 15mM NaHCO3, 15mM hepes, 33 μM biotin, 17μM pantothenate, 0.67μM human insulin, 0.2mM triiodothyronine, 0.5mM dexamethasone (DEX) and antibiotics for 21 days. Within 15-21 days, cells have achieved maximum differentiation. Cells were regarded as differentiated by morphological criteria when, after acquiring a round shape, their cytoplasm was completely filled with multiple lipid droplets as assessed by Oil Red O staining. The proportion of differentiated cells is estimated by direct counting under the microscope of total and differentiated cells, using a micrometer.

The 3T3-L1 fibroblasts were grown in DMEM with 10% FCS. T47D cells were initially grown in RPMI 1640 with 10% FCS containing 0.02mM hepes, whereas MCF7 cells were grown in MEM with 10% FCS until confluent. SSC02, SC78 and SSC30 breast cancer cells (kindly provided by Dr. Adi Gazdar) were grown to confluence in DMEM with 10% FCS. All cells were incubated at 37°C, in 5% CO2. To induce the adipogenic differentiation of 3T3-L1 fibroblasts within 2 days of reaching confluence, these cells were treated with DEX (0.25 μm), MIX (0.5 mM) and insulin (1μg /ml) for 2 days and then maintained in DMEM with 10% FCS maintained for 6 additional days. Cells containing multiple fat droplets were scored as differentiated by phase contrast microscope after staining with Oil Red O. All culture media were phenol red free.

Co-cultures of 3T3-L1 fibroblasts with breast cancer and human adipose fibroblasts with breast cancer cells were performed using 35mm 6-well plates. 3T3-L1 cells or adipose fibroblasts were plated on the bottom wells. Breast cancer cells were seeded on the permeable membrane (0.45μm) tissue culture inserts. 3T3-L1 were cultured in DMEM with 10% FCS, after they reach confluence for two days. The medium was then changed to the appropriate differentiation medium for 48 hours. Then the medium was switched back to DMEM plus 10% FCS for 6 days. In the case of human adipose fibroblasts, co-cultures were maintained for 15-20 days in the differentiating medium, since these cells required prolonged exposure to this medium in contrast to 3T3-L1 murine fibroblasts.

At the end of coculture experiments, human adipose fibroblasts or 3T3-L1 cells in the bottom plate were evaluated for differentiation to mature adipocytes and for proliferative indices (rate of [^{3}H]thymidine incorporation and flow cytometry) or were harvested for RNA isolation. Anti-human IL-11 (Ab-218-NA) and TNFα (Ab-210-NA) neutralizing antibodies were purchased from R&D Systems, Inc. (Minneapolis, MN).

Immunohistochemistry

Anti-human IL-11, TNF, C/EBPα, C/EBPβ and C/EBPδ antibodies were purchased from the R&D systems, Inc. (Minneapolis, MN). The immunohistochemical procedures were performed, as previously described, on 2.5μm-thick sections mounted on poly-L-lysine-coated slides using the biotin-streptavidin amplified technique with a Histone immunostaining kit (Nichirei, Tokyo, Japan). Briefly this staining procedure was performed as follows: 1) routine deparaffinization; 2) inactivation of endogenous peroxidase activity with 0.3% H_2O_2 in methyl alcohol for 20 minutes at 23°C; 2) blocking with 1% goat serum for 45 minutes at 23 C; 4) incubation with the primary antibody at 4C for 18 h; 5) incubation with biotinylated goat anti-rabbit antibody for 30 minutes at 23C; 6) incubation with peroxidase-conjugated streptavidin for 30 minutes at 23°C; 7) colorimetric reaction with a solution containing 0.05% Tris-HCI ([H 7.6), 0.66 mol/L 3,3'-diaminobenzidine and 2 mol/L H_2O_2 and 8) counterstaining with 1% methyl green.

RESULTS

Effects of tumor cells on differentiation of fibroblasts to adipocytes

Confluent 3T3-L1 cells differentiate to mature adipocytes, within 4 or 6 days after a 48-hour treatment with the cocktail including insulin, DEX and MIX. At this stage, the cells appear rounded and possess numerous large cytosolic lipid spheres as revealed by Oil Red O staining (Fig. 1A). The effect of T47D breast cancer cell line on the differentiation in the 3T3-L1 cells was evaluated with co-cultures or T47D cell-conditioned medium. T47D breast cancer cells completely inhibited the differentiation of 3T3-L1 cells (Fig. 1B). Upon addition of neutralizing antibodies to human IL-11 and TNFα, this effect was totally reversed. In other words, neutralizing both of these cytokines reversed the inhibitory effect of breast cancer cells on adipocyte differentiation (Fig. 1C). This reversal was partial, when either antibody was used separately and was dose-dependent (data not shown).

Cocultures of T47D cancer cells also inhibited the differentiation of human adipose fibroblasts completely. Other breast cancer cell lines (MCF7, SSC202, SSC78, SSC30) also inhibited the differentiation of both 3T3-L1 murine fibroblasts and human adipose fibroblasts. Moreover, the liver cancer cell line HepG2 and the choricarcinoma cell line JEG3 also inhibited the differentiation of these cells to adipocytes (data not shown).

Expression of PPARγ, C/EBPα and aP2 in 3T3-L1 cells cocultured with breast cancer cells.

We determined the levels of transcripts of C/EBPα, C/EBβ, C/EPBδ, PPARγ and aP2 by northern analysis in confluent 3T3-L1 cells exposed to the adipocytic differentiation cocktail only (DMEM + COC), the cocktail plus the T47D cell-conditioned medium (CM + COC) or simple culture medium only (DMEM used as a control). Figure 2 (A and B) depicts these results. TCM specifically decreased the transcript levels of C/EBPα and PPARγ in 3T3-L1 cells but not those of C/EBβ and C/EPBδ. As expected, TCM also inhibited the expression of aP2, which is a marker for adipocyte differentiation (Figure 2B). These results were confirmed by immunohistochemistry.

We determined the number and staining intensity of immunoreactive cells for C/EBPα, C/EBβ and C/EPBδ in fibroblasts mixed with malignant epithelial cells (intratumoral), within fat 1 cm from the tumor (adjacent) and within fat 2-4 cm from the tumor (distant). Figure 3 (A-G) illustrates fibroblasts and adipocytes with immunoreactive nuclei for these transcription factors. C/EBPα was not detectable in intratumoral fibroblasts, but it was readily detectable in fibroblasts and adipocytes in adjacent and distant fat tissue biopsies from 10 patients. No differences were observed in the distribution of expression of C/EBβ and C/EPBδ. An H-scoring system was used to determine the number of immunoreactive fibroblasts, and this is illustrated in Figure 4. These results are in agreement with those of the northern analysis (Figure 2).

Cellular localization of the cytokines inhibitory for adipocyte differentiation in breast cancer

Previous experiments have suggested that IL-11 and TNFα (Figure 1C) mediated the inhibition of adipocyte differentiation by cancer cells. Thus, we determined the distribution of immunoreactive IL-11 and TNFα in 15 mastectomy specimens. These two cytokines were primarily expressed in malignant epithelial cells. Less than 25% of fibroblasts were immunoreactive showing considerably less staining intensity (Figure 5A-B).

Effects of T47D breast cancer cells on the proliferation of 3T3-L1 fibroblasts

We determined whether T47D cancer cells affect the proliferation indices of 3T3-L1 fibroblasts when co-cultured. Figure 6 demonstrates no differences in the DNA histograms of 3T3-L1 cells incubated in the absence or presence of T47D cells using flow cytometry. Neither did we see an effect of T47D breast cancer cells on the [^{3}H]thymidine incorporation into 3T3-L1 cells. Thus, we conclude that breast cancer cells induce accumulation of fibroblasts in the tumor tissue by the inhibition of differentiation of these cells to mature adipocytes but not by promotion of their proliferation.

DISCUSSION

Peri- and intratumoral fibroblasts provide structural support to tumor tissue, and secretory products of fibroblasts may promote tumor growth. We herein demonstrated that malignant breast epithelial cells actively participate in the process of accumulation of stromal fibroblasts in and around the tumor tissue (*i.e.* desmoplastic reaction). Secretory products of cancer cells prevent the differentiation of fibroblasts to adipocytes and, in fact, may even reverse adipocyte differentiation. We also demonstrated that tumor-derived cytokines act on adjacent adipose stroma by downregulating the expression of adipogenic factors such as the C/EBPα and PPARγ. These findings are in contrast with what has been classically viewed as the mechanism of desmoplastic reaction in the breast cancer (*i.e.* the proliferation of fibroblasts). This study provides evidence that breast cancer cells (or their secretory products) do not induce proliferation of fibroblasts. Thus, inhibition of differentiation seems to be the major mechanism responsible for desmoplastic reaction.

Differentiation of fibroblastic cells to adipocytes appears to be primarily controlled by a cascade of adipogenic factors. C/EBPβ and C/EPBδ appear to mediate the earlier phase of the differentiation program. PPARγ, a nuclear hormone receptor, is expressed next in the differentiation process and becomes adipogenic after binding to its synthetic (BRL49653) (26) and natural (15-deoxy-$\Delta^{12,14}$-prostaglandin J_2) ligands (23). PPARγ binds to its response elements (PPREs) in the promoters of its target genes. To recognize a PPRE, PPARγ must form a heterodimer with RXRα, a basic-leucine zipper transcription factor. On the other hand, C/EBPα is not expressed until relatively late in the differentiation process. It binds to and transactivates the promoters of a number of adipocyte genes. Thus, breast cancer cells seem to exert their inhibitory effect on relatively later stages of the differentiating process, namely inhibition of the expression of PPARγ and C/EBPα. This is consistent with the isolated effects of TNFα, which has been shown to inhibit the expression of PPARγ and C/EBPα in 3T3-L1 cells and fetal brown adipocytes (27,28).

This report lays the groundwork for the mechanism of desmoplastic reaction as an epithelial-stromal interaction in the breast cancer. Further studies are required to identify other key molecules in the cancer-mediated inhibition of adipocyte differentiation. Two candidate substances are CHOP, a transcription factor that acts as a negative dominant regulator of adipocyte differentiation and Pref-1, which is a transmembrane protein with EFG-like motifs and another negative regulator of adipocyte differentiation (29). The determination of the roles of these candidate substances will further increase our understanding of the epithelial-stromal interactions in the breast cancer.

FIGURE LEGENDS

Figure 1.

Panel A: 3T3-L1 cells, 8 days after reaching confluence. Confluent cells were treated with the differentiating cocktail (dexamethasone, insulin and MIX) for the first 2 days and then were maintained in DMEM for the following 6 days. Accumulation of lipid droplets represented by the red stain was detected in the cytoplasms of 80% of adipocytes (stain: Oil Red O).

Panel B: Coculturing of 3T3-L1 fibroblasts with breast cancer cells (in this instance, T47D cells) completely inhibited their differentiation to adipocytes evident by the lack of lipid droplets.

Panel C: This cancer-induced inhibition of differentiation was totally reversed by the addition of neutralizing antibodies to IL-11 and TNFα. This indicates that cancer cells inhibit adipocyte differentiation, at least in part, by the secretion of IL-11 and TNFα.

Figure 2. Northern blot showing the levels of transcripts of C/EBPα, C/EBPβ, C/EBPδ (Panel A), PPARγ and aP2 in 10 μg of total RNA from 3T3-L1 cells incubated under conditions explained in the legend of Figure 1. The 28S RNA fraction was included to demonstrate the presence of comparable amounts of RNA in each lane. (DMEM: control medium; COC: differentiating cocktail; TCM: T47D cell-conditioned medium). T47D cells (TCM) inhibited expression of C/EBPα and PPARγ but not of C/EBPβ or C/EBPδ in 3T3-L1 cells after exposure to the differentiating cocktail (COC). The expression of aP2, a marker of differentiated adipocytes, was also suppressed by T47D cells (TCM).

Figure 3.

Panel A: Immunoreactive C/EBPα was not detected in the nuclei of stromal (adipose) fibroblasts within the breast tumor (intratumoral). Brown stain indicates C/EBPα.

Panel B: C/EBPα was readily detectable in adipose fibroblasts/adipocytes distant to the tumor evident by the lack of malignant epithelial cells.

Panel C: C/EBPβ was readily detectable in the nuclei of intratumoral stromal (adipose) fibroblasts (brown stain).

Panel D: C/EBPβ was readily detectable in the nuclei of adipose fibroblasts/adipocytes distant to the tumor.

Panel E: C/EBPδ was readily detectable in the nuclei of intratumoral stromal (adipose) fibroblasts (brown stain).

Panel F: C/EBPδ was readily detectable in the nuclei of adipose fibroblasts/adipocytes distant to the tumor.

Figure 4. Graphic representation of the data in Figure 3. Immunoreactive proteins were scored in the 3 biopsies (intratumoral, adjacent fat, distant fat) from each of the 10 mastectomy specimens. H-scoring was used in the following manner:

0: 0 - 5% Fibroblasts are positive
1: 6 - 25% Fibroblasts are positive
2: 26 – 50% Fibroblasts are positive
3: 51 – 75% Fibroblasts are positive
4: 76 – 100% Fibroblasts are positive

Expression of C/EBPα in intratumoral fibroblasts were found to be lower compared with fibroblasts in cancer-free breast adipose adjacent or distant to the tumor.

Figure 5.

Panel A: Immunoreactive TNFα (brown stain) is readily detectable in malignant epithelial cells but not in fibroblasts.

Panel B: Immunoreactive IL-11 (brown stain) is readily detectable in malignant epithelial cells but not in fibroblasts/adipocytes.

Figure 6. No differences in the DNA histogram were detected in 3T3-L1 cells incubated without (A) or (B) with T47D breast cancer cells. The numbers of cells in G_0/G_1, S and G_2 were not significantly different.

Figure 7. Results of [^{3}H]thymidine uptake by 3T3-L1 incubated under various conditions.

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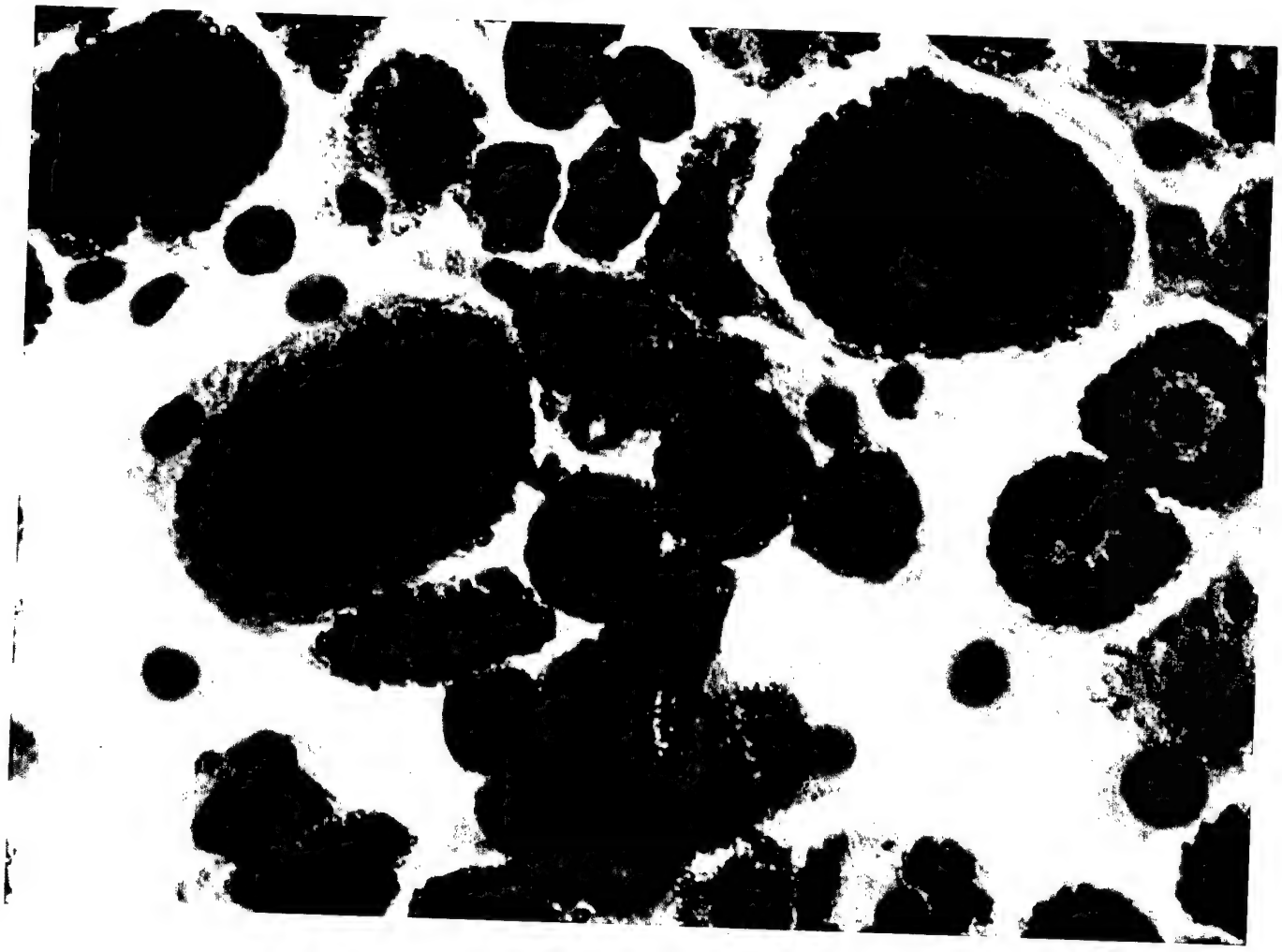

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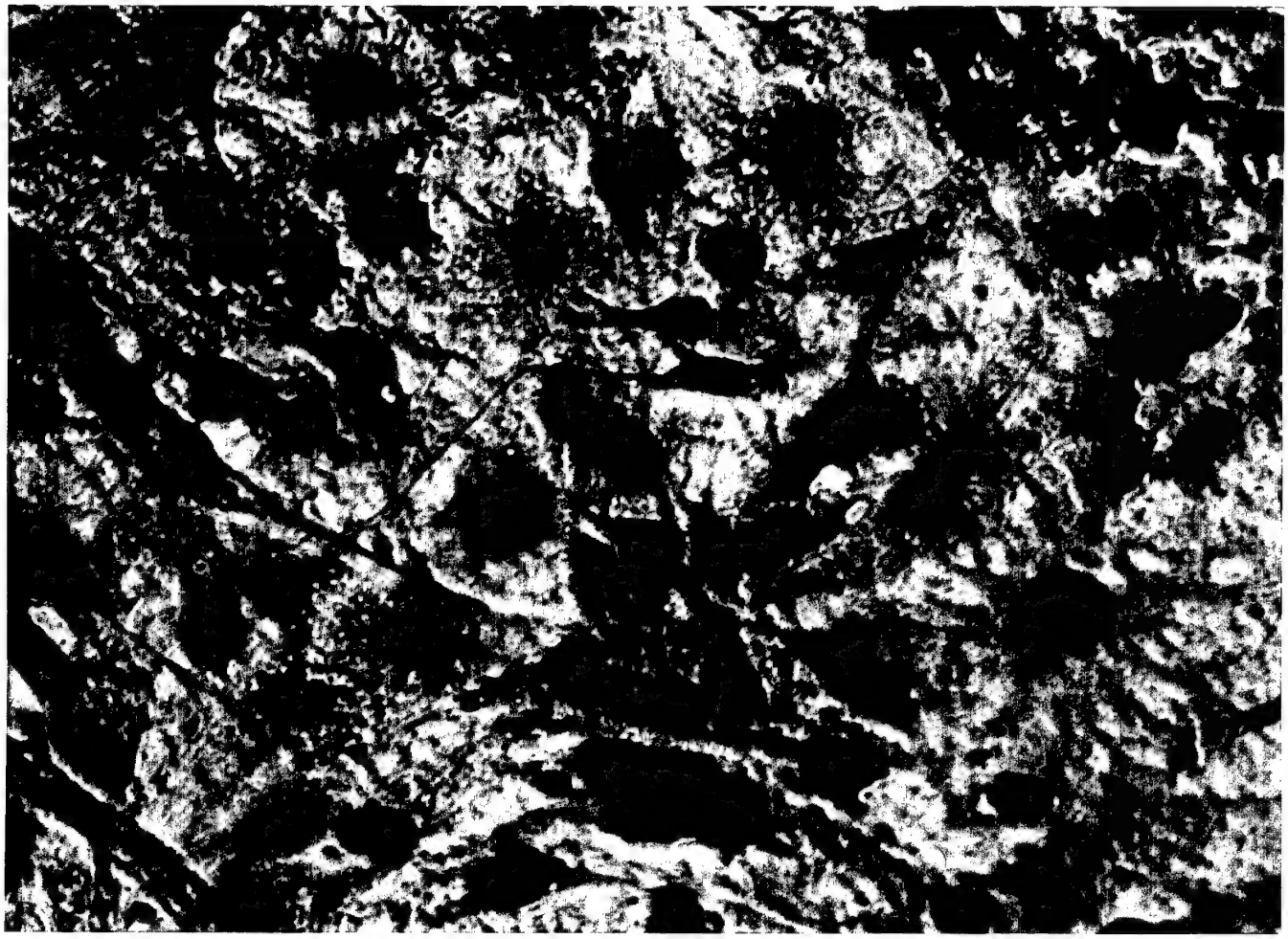

Figure 1B

Figure 1C

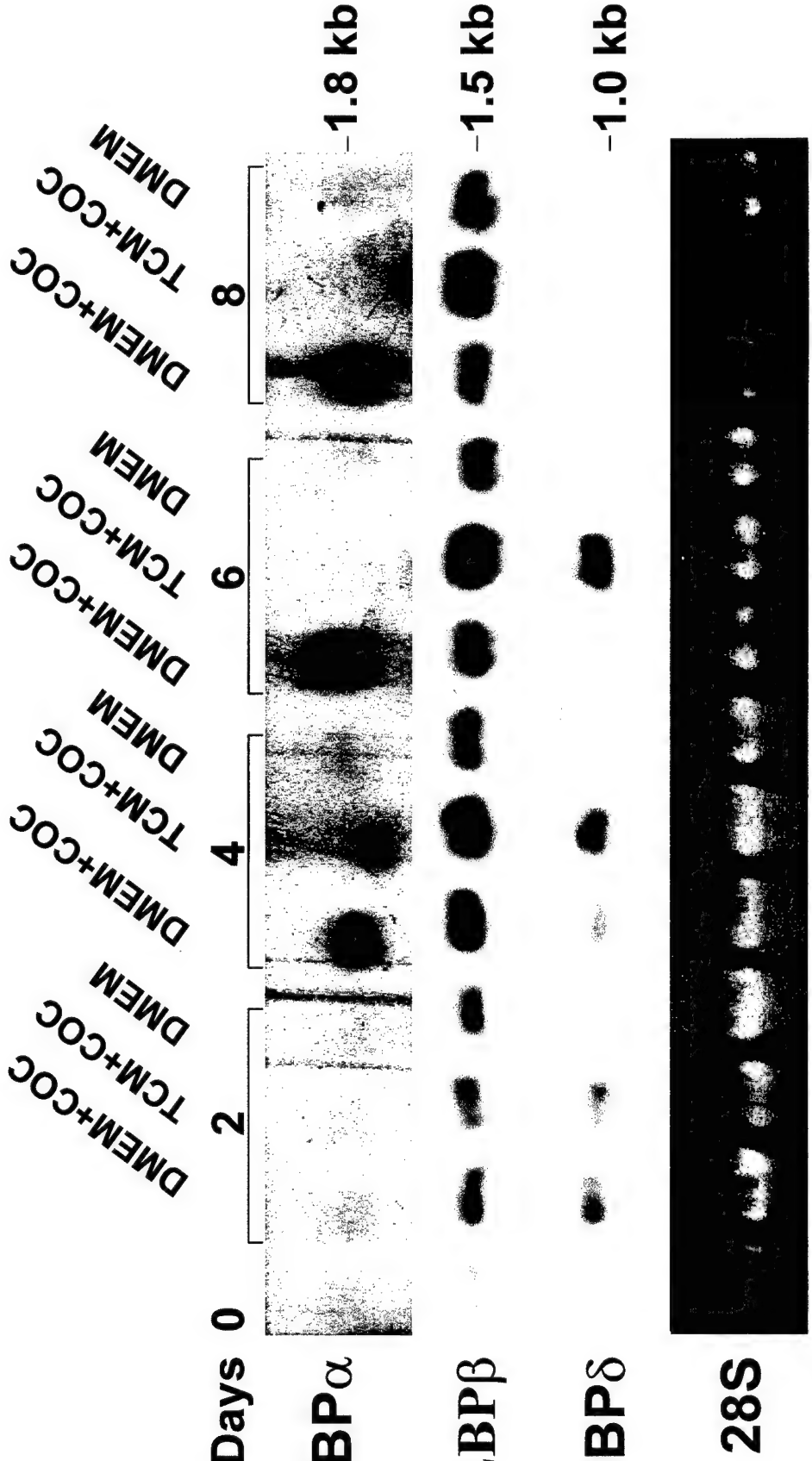
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Figure 2A

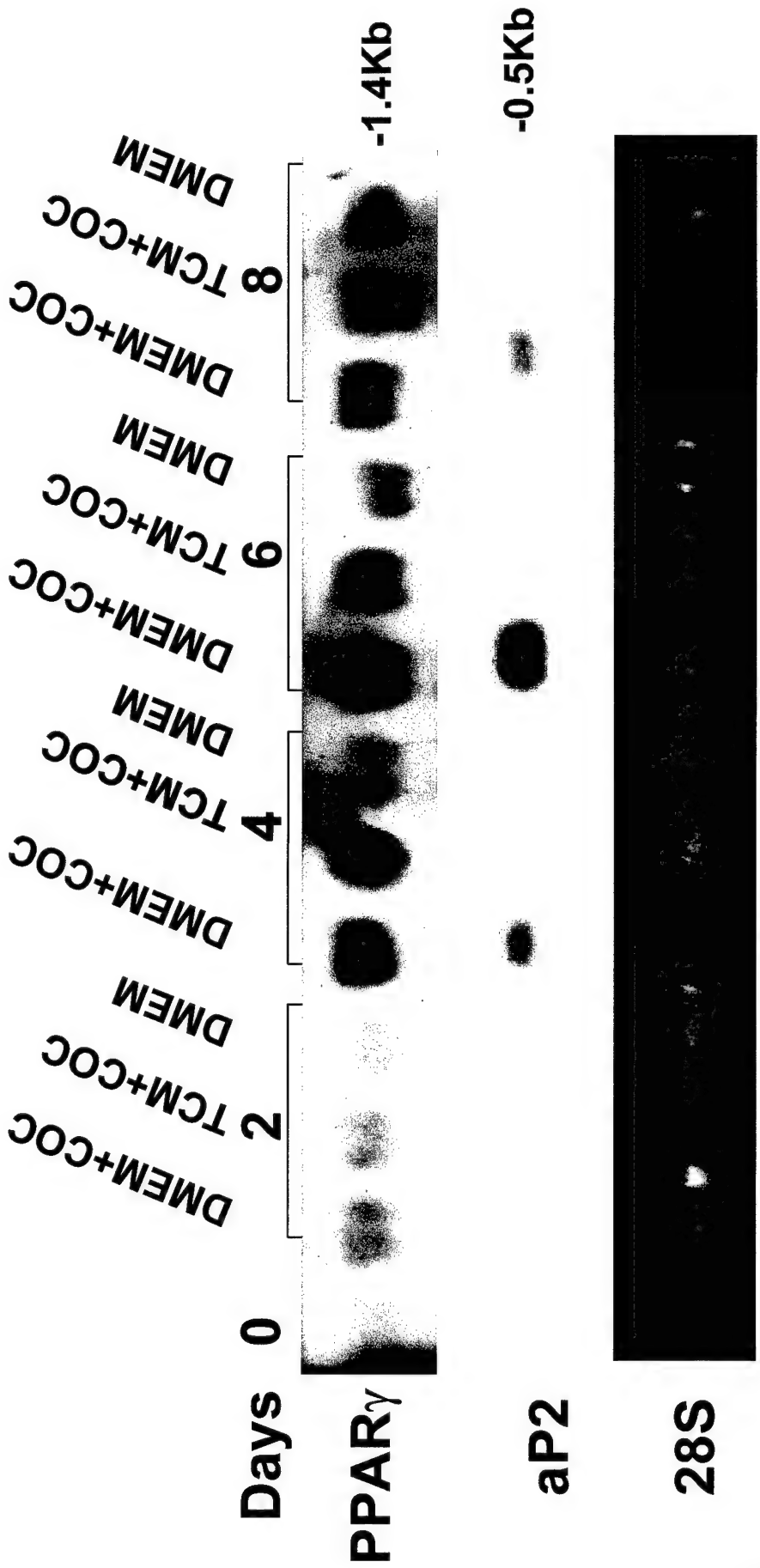
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Figure 2B

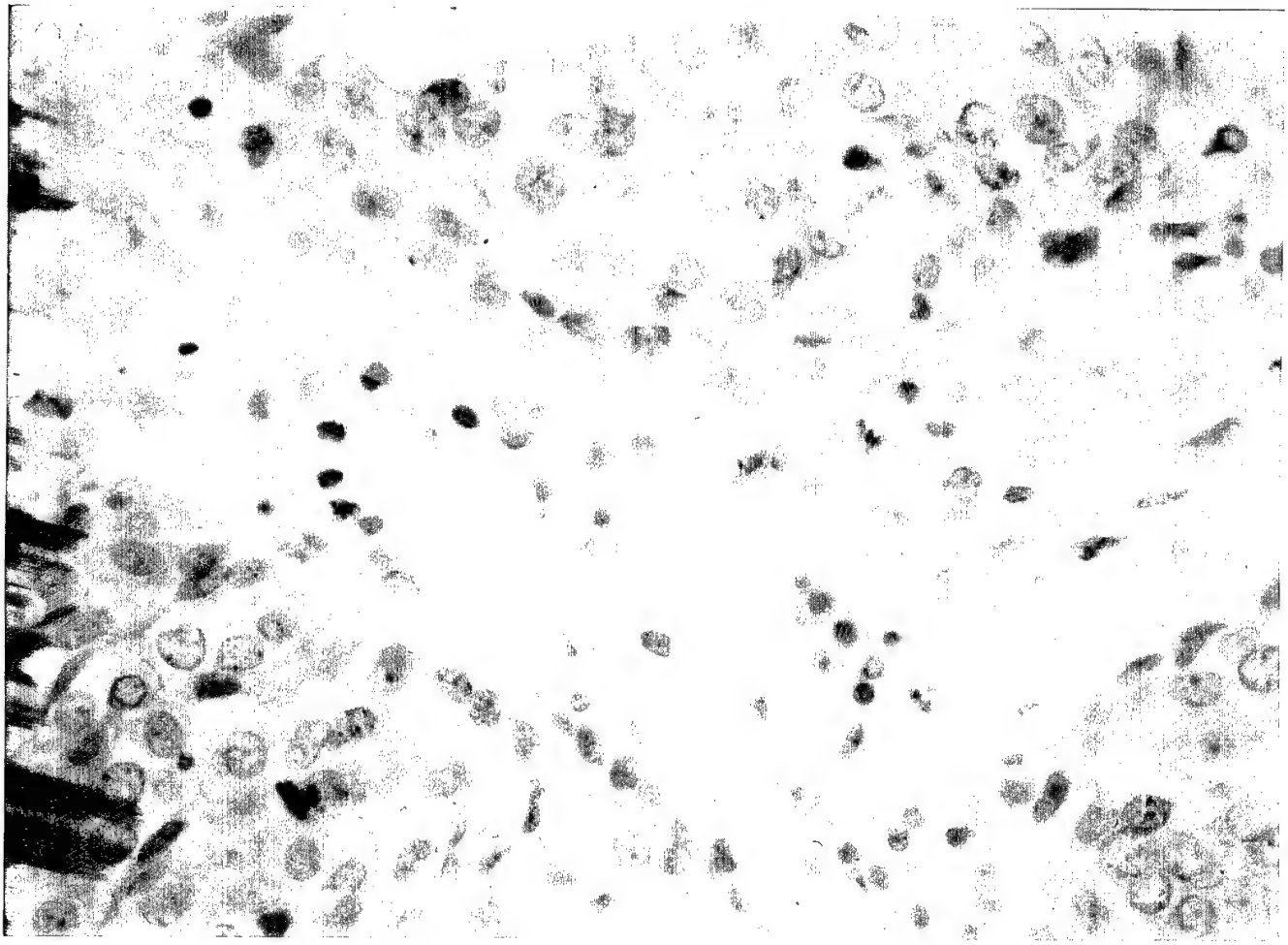
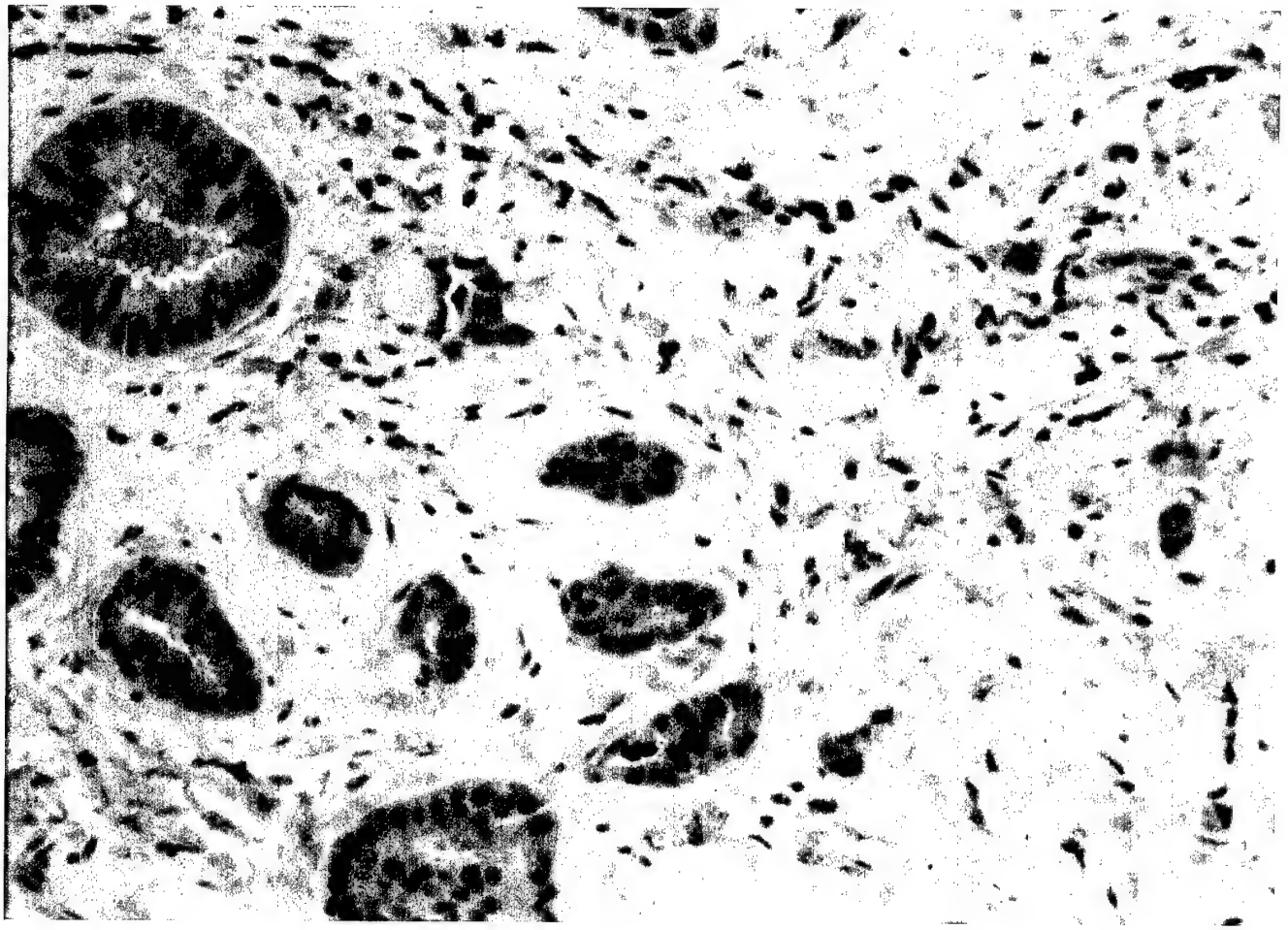


Figure 3A

Figure 3B



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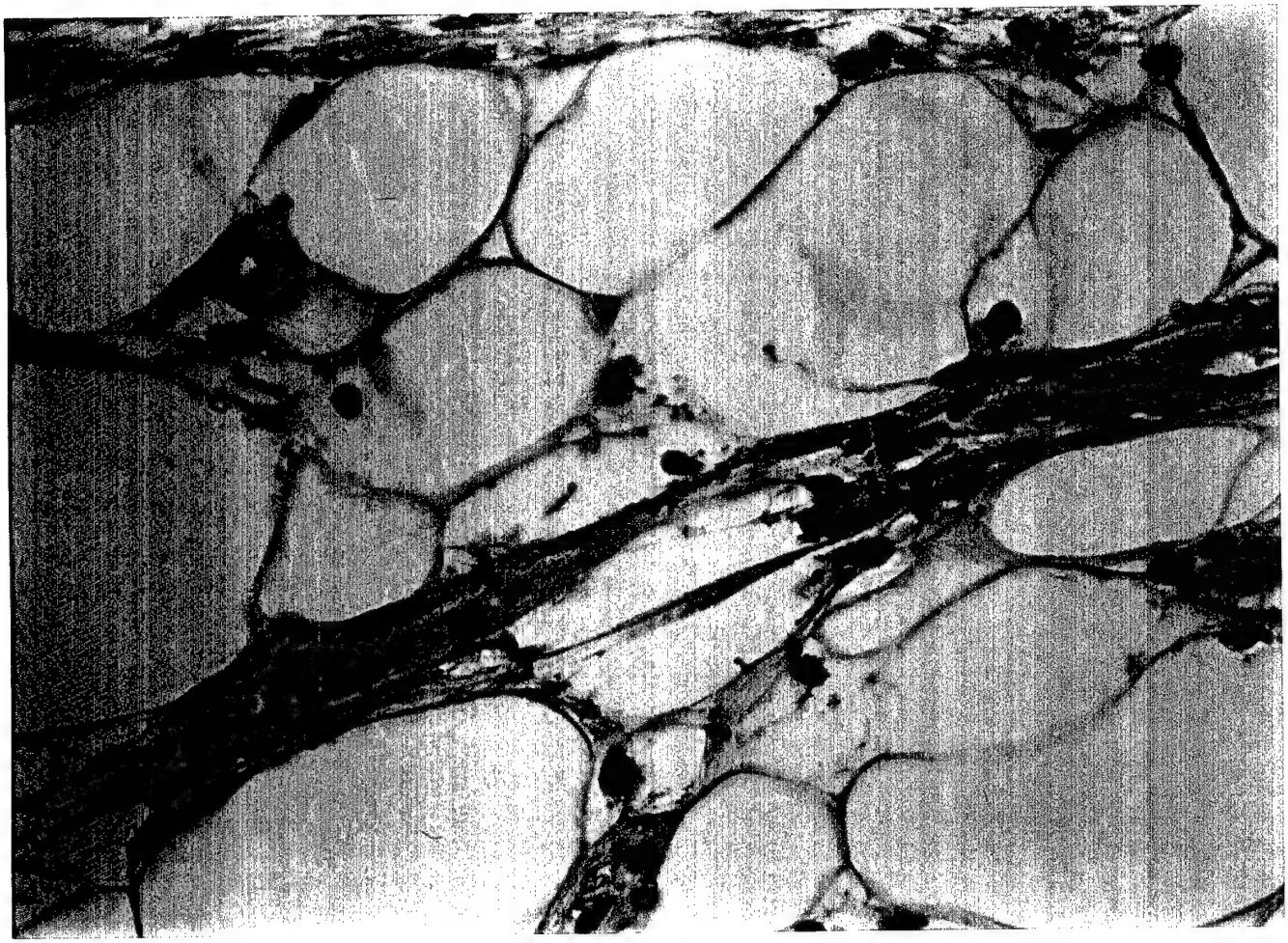

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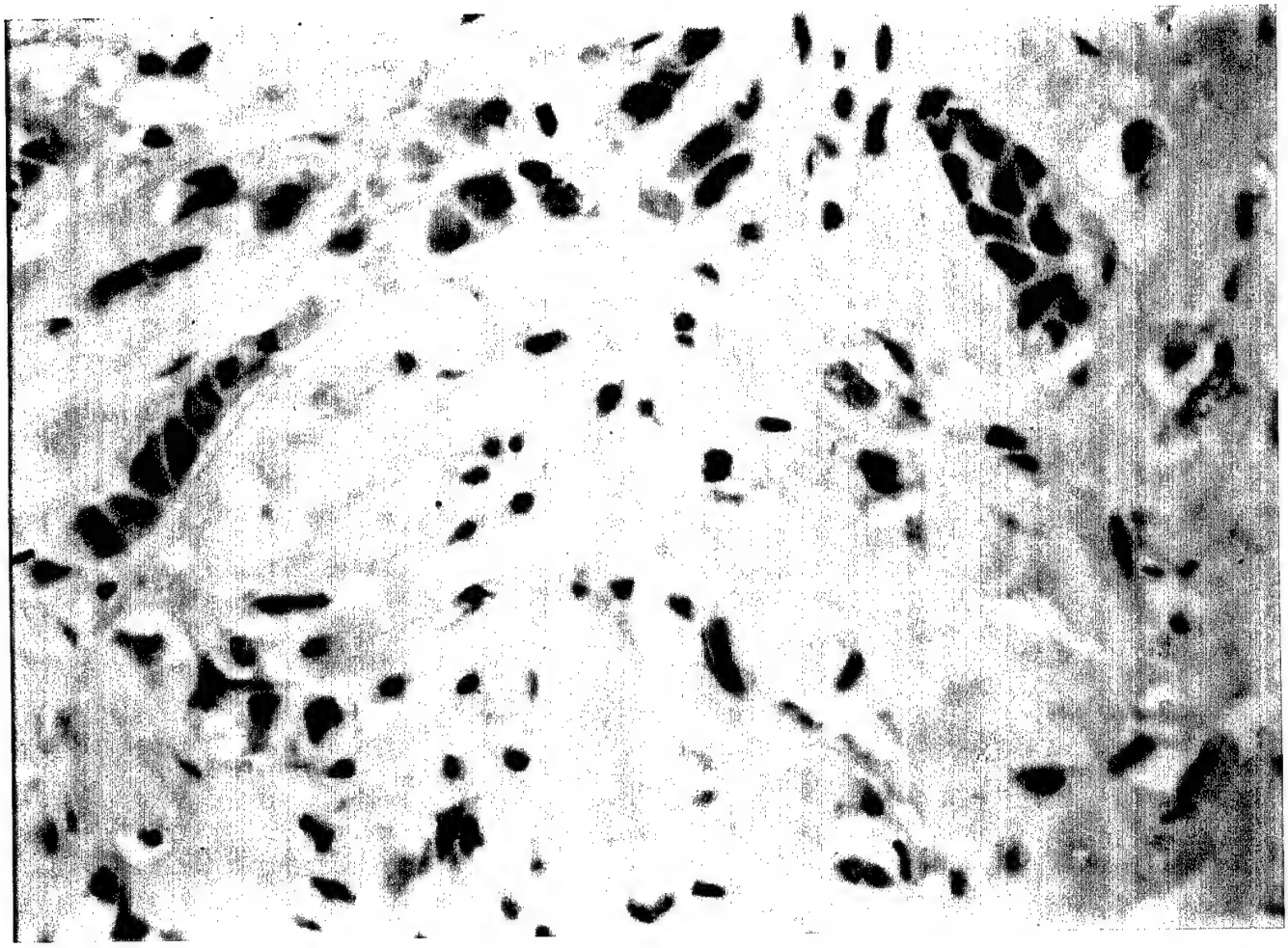

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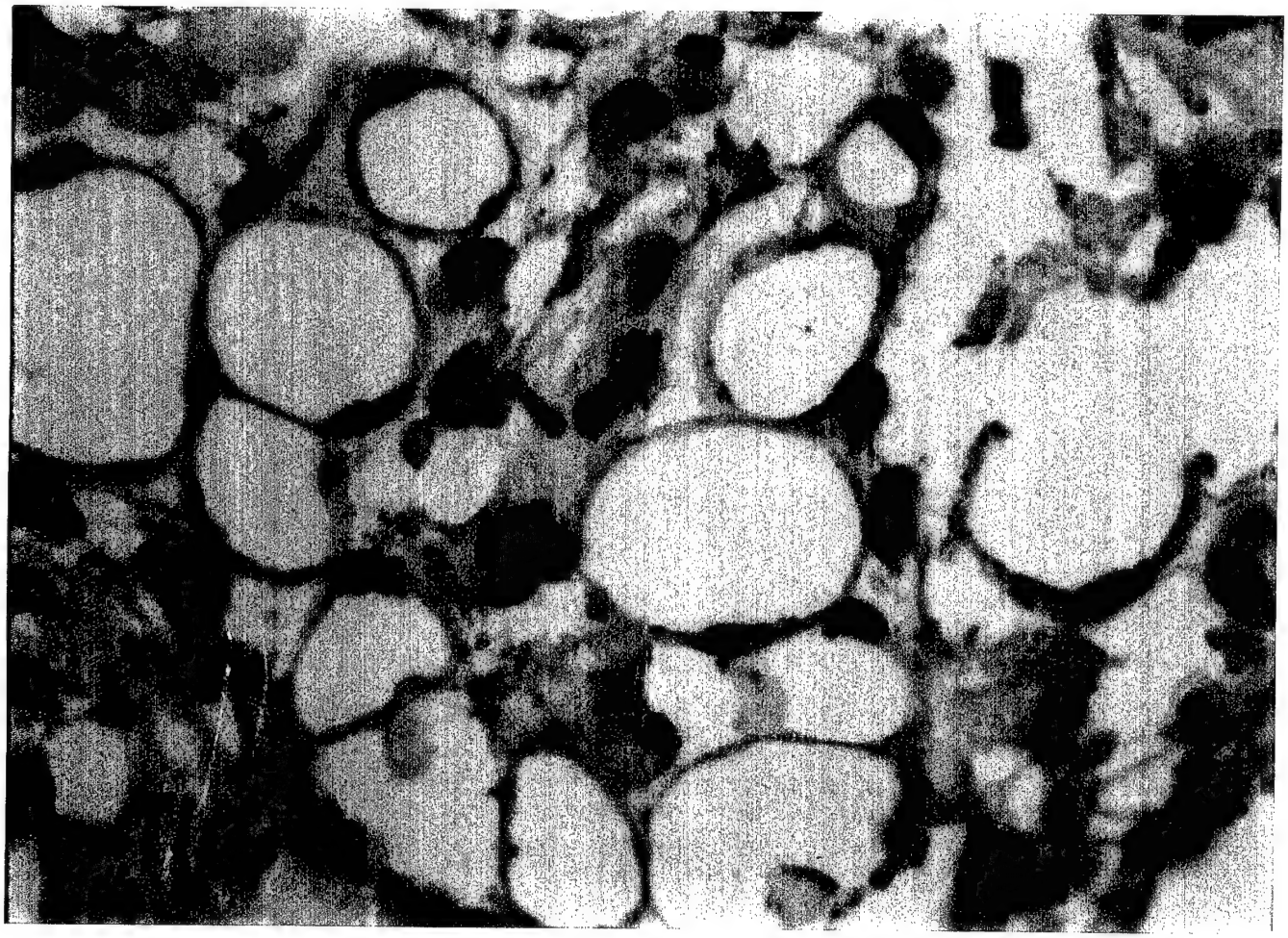

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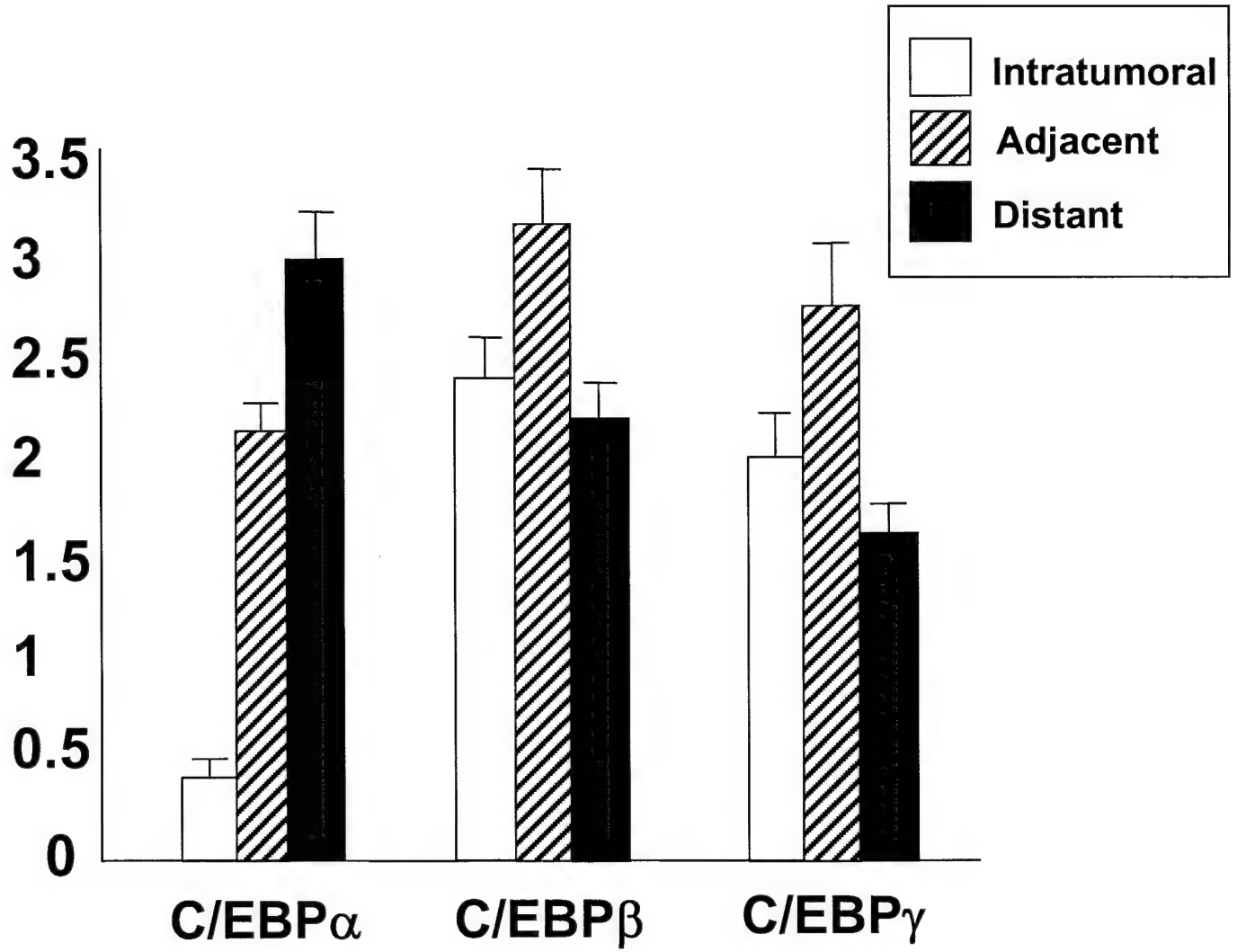
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C/EBPγ

Figure 4

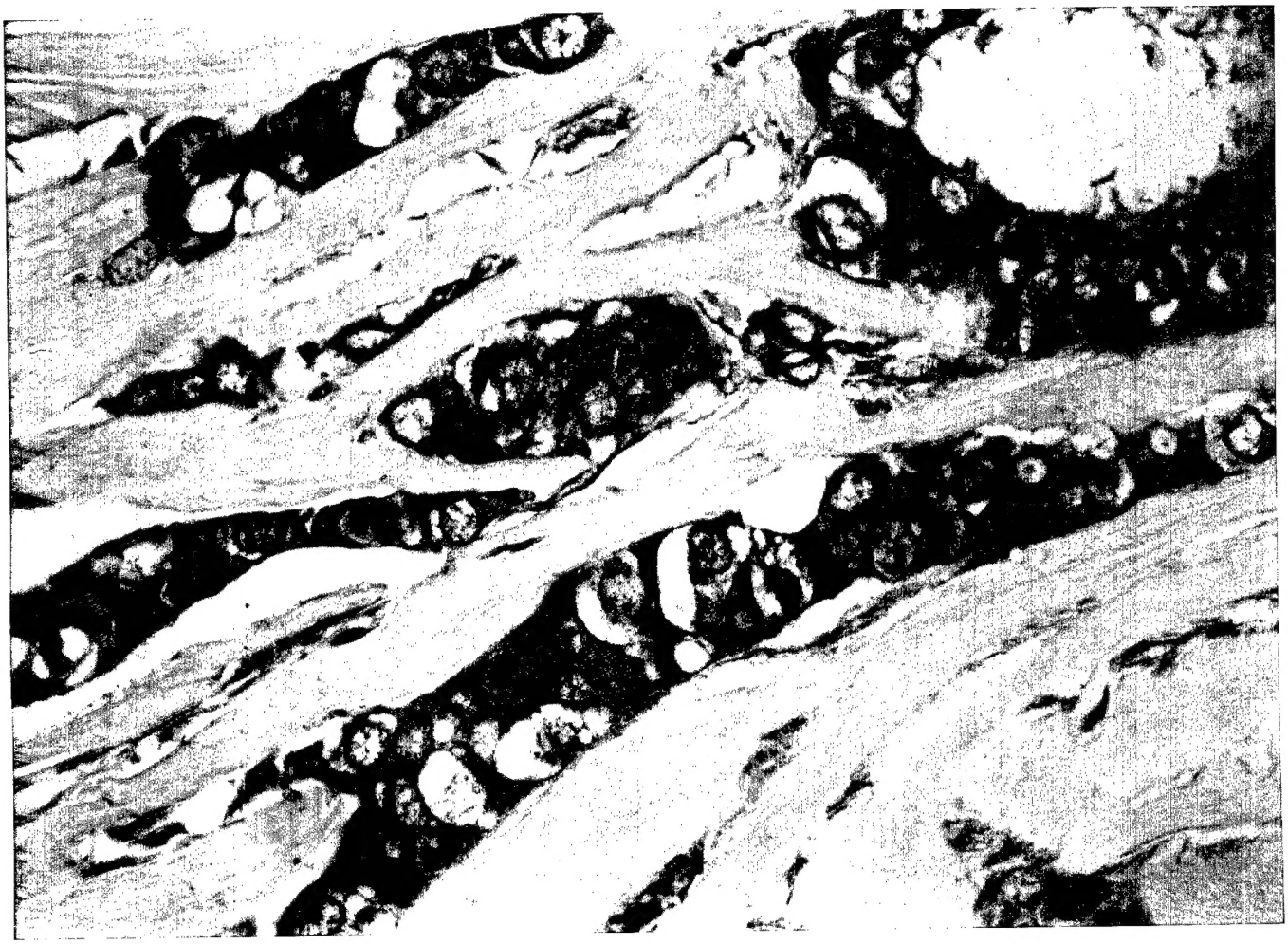


Figure 5A

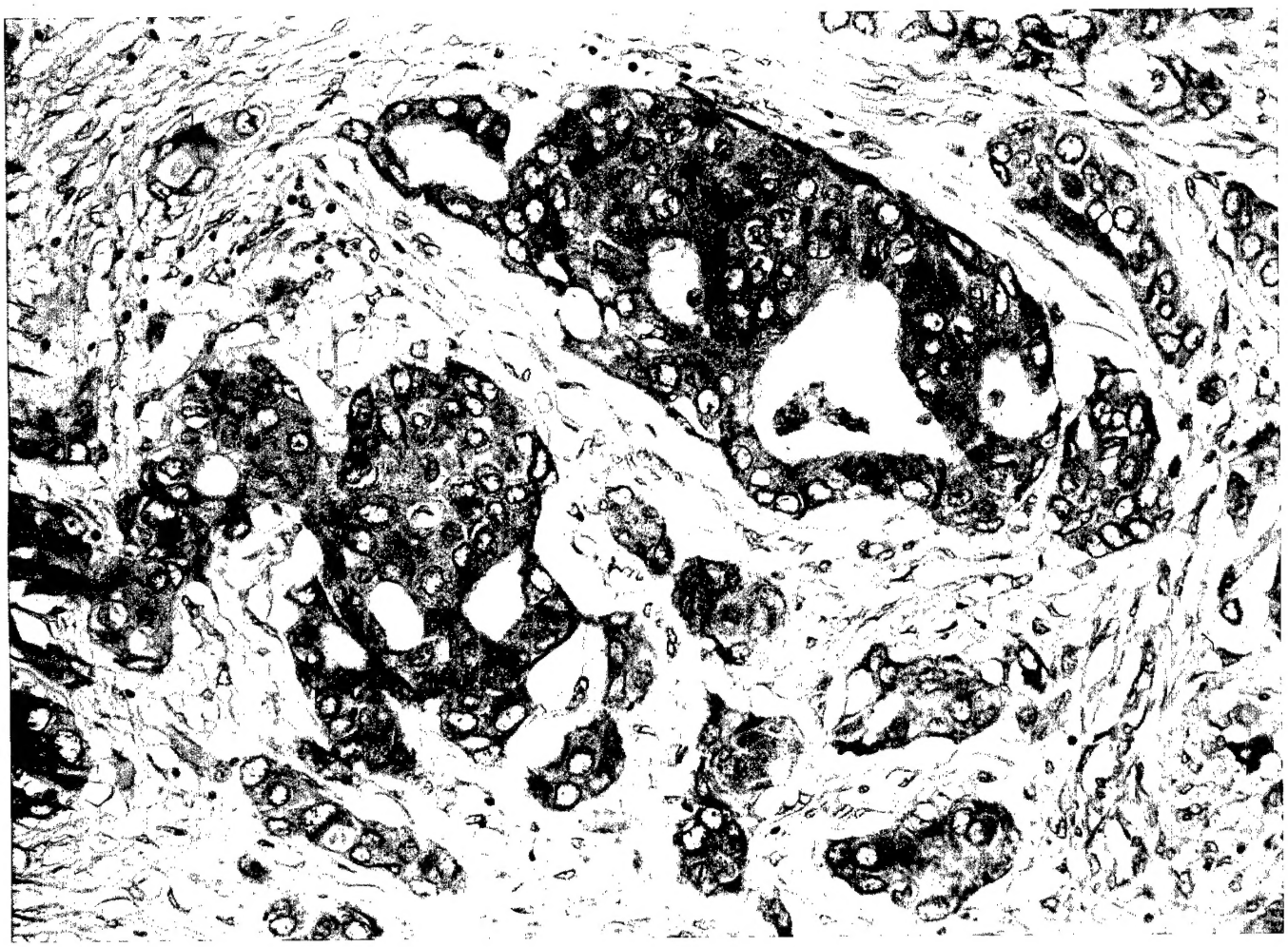
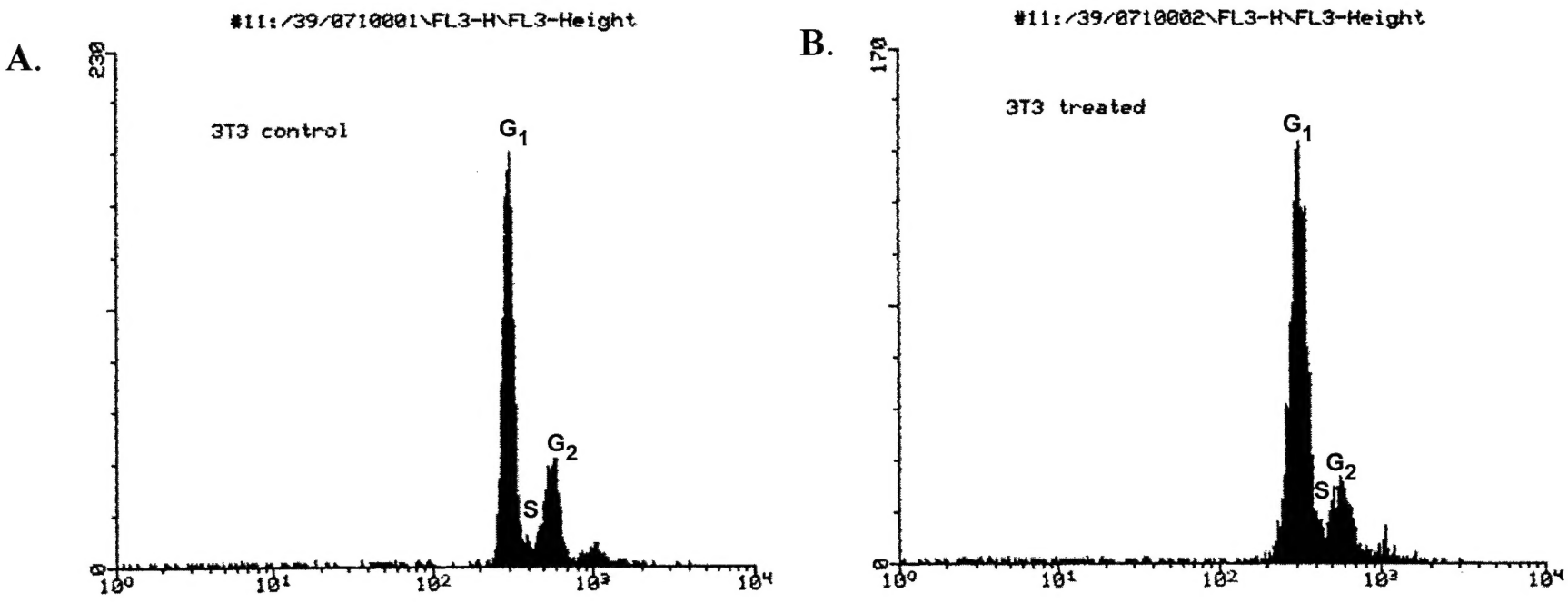
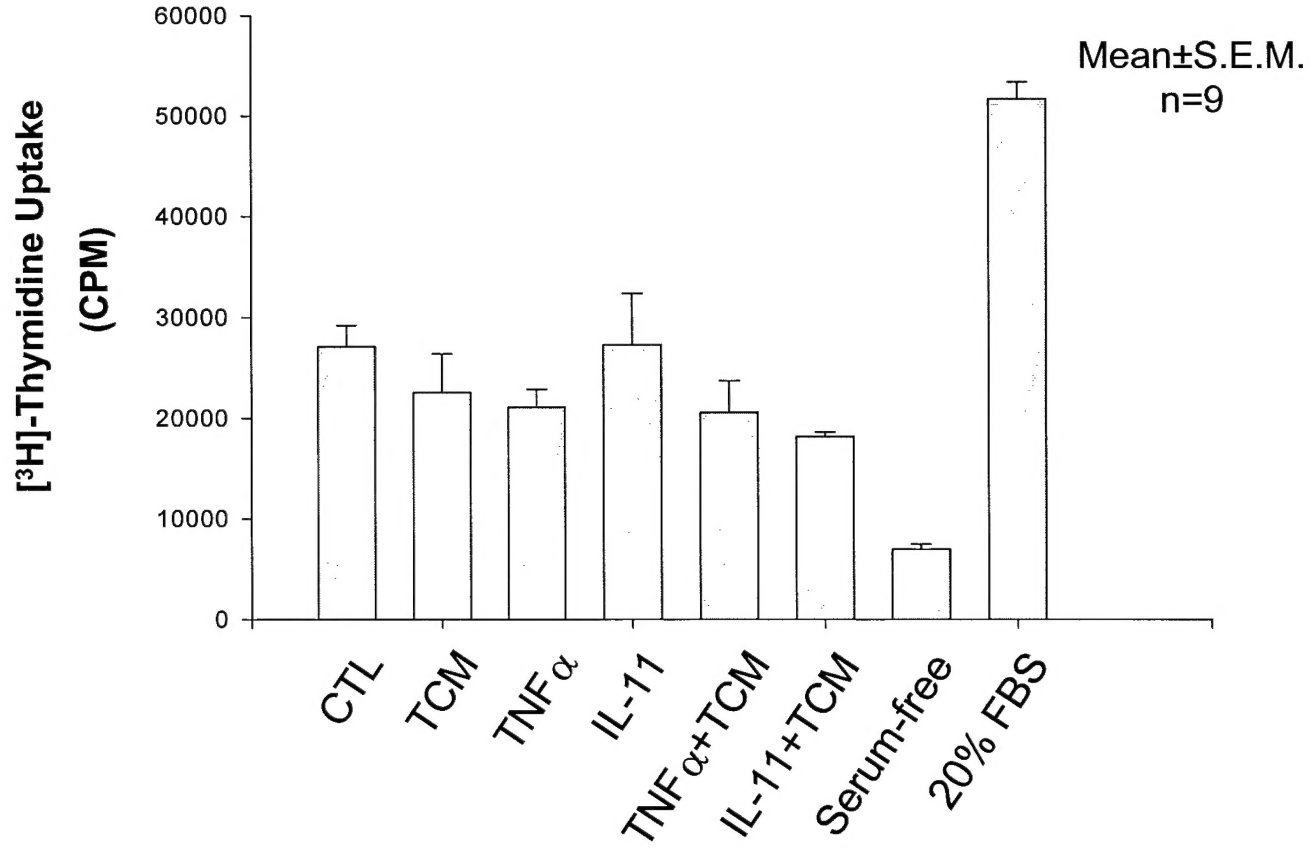


Figure 5B



Flow cytometry analyses of 3T3-L1 cells maintained in culture medium (DMEM) only (A), and cells treated with T47D breast cancer cell-conditioned medium (B). No differences were noted in the number of cells in various phases of cell cycle.

Figure 6



Assay of [^{3}H]-thymidine uptake to determine whether T47D breast cancer cell-conditioned medium (TCM) increases 3T3-L1 cell proliferation . 3T3-L1 cells were plated as 20% confluent in DMEM with 0.1% fetal bovine serum (FBS) and incubated under different conditions for 24h . During the last 18h of incubation, [^{3}H]-thymidine was added . Incubation with TCM or cytokines did not affect the proliferation of 3T3-L1 cells . Serum-free medium (control, CTL) and medium with 20% FBS were included as controls .

Figure 7